microRNA in Macrophage Polarization and Spinal Cord Injury

Undergraduate Thesis

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Abstract

Spinal cord injury (SCI) is a leading cause of disability among young adults. SCI is the result of trauma to the spinal cord, and most commonly occurs due to motor vehicle accidents. Treatment options for SCIs are limited and often have very serious consequences. The recruitment and activation of innate immune cells at the site of injury affects recovery. Macrophages with a pro-inflammatory phenotype (M1) persist at the site of injury and are known to cause further damage. These macrophages are important for the removal of debris and damaged tissue and the further recruitment of peripheral immune cells to the site of injury. Conversely, macrophages with a regenerative, anti-inflammatory phenotype (M2) are only transiently located at the site of SCI. miRNAs are important regulators of inflammation in autoimmune and inflammatory disorders. miRNA are small, non-coding RNAs, approximately 19-24 nucleotides in length, which regulate gene expression at the transcriptional level. miRNA can bind messenger RNA (mRNA) through base pairing and induce the degradation of mRNA or inhibit its translation. Therefore, we hypothesized that miRNA play a key role in the M1 vs M2 macrophage differentiation process. To identify miRNA involved in this process, the relative expression of several miRNA involved in inflammatory responses was analyzed *in vitro* in bone marrow derived macrophages (BMDM) undergoing differentiation into M1 versus M2 phenotypes. We found significantly higher expression levels of one miRNA in particular, miR-155, in BMDM undergoing M1 differentiation, as compared to BMDM undergoing M2 differentiation. miR-155 knockout (KO) macrophages were unable to efficiently up-regulate inducible nitric oxide synthase (iNOS), a characteristic marker of M1 macrophages, as well as the inflammatory cytokine TNF-α. Furthermore, the expression of arginase-1 (Arg1), a prototypical M2 marker, was stable or increased in miR-155 KO macrophages. These results support the concept that miR-155 plays an important role in the differentiation of macrophages into the M1 phenotype and help lay a framework for understanding the mechanism(s) by which miRNA influence macrophage differentiation and inflammation after SCI.

Abbreviations

Arg-1	Arginase-1
AP-1	Activation Protein-1
BBB	Blood Brain Barrier
BMDM	Bone Marrow-Derived Macrophages
CD	Cluster of Differentiation
CNS	Central Nervous System
CSPG	Chondrotin Sulfate Proteoglycan
DAMPs	Danger-Associated Molecular Patterns
FC	Fold Change
HMGB1	High Mobility Group Box-1
IFN	Interferon
IL	Interleukin
iNOS	Inducible Nitric Oxide Synthase
IRAK-1	IL-1 Receptor Associate Kinase-1
IRF	Interferon Response Factor
JAK	Janus Kinase
KO	Knock-out
LPS	LipoPolySaccharide
M&M	Materials and Methods
M0	Undifferentiated macrophages
M1	Classically activated macrophages
M2	Alternatively activated macrophages
MAG	Myelin Associated Glycoprotein
MHCII	Major Histocompatibility Complex Class II
miRNA	MicroRNA
MMPs	Matrix MetalloProteinases
NF-κB	Nuclear Factor – Kappa B
NO	Nitric Oxide
PAMPs	Pathogen-Associated Molecular Patterns
Pre-miRNA	Precursor miRNA
Pri-miRNA	Primary miRNA
PRRs	Pattern Recognition Receptors
RISC	RNA-Induced Silencing Complex
SCI	Spinal Cord Injury
SOCS	Suppressor of Cytokine Signaling
STAT	Signal Transducer and Activator of Transcription
TGFβ	Transforming Growth Factor beta
TLR	Toll-Like Receptors
TNFα	Tumor Necrosis Factor alpha
UTR	UnTranslated Region
VEGF	Vascular Endothelial Growth Factor

I Background

1.1 - Introduction

Spinal cord injury (SCI) is damage of the spinal cord that results from trauma as opposed to disease [1]. Although patients who survive the first 24 hours post-injury generally have a good survival rate, SCI has severe long-term consequences. Depending on the location and severity of trauma, paraplegia (paralysis of two limbs) and quadriplegia (paralysis of four limbs) are common outcomes of SCI. There are few treatment options, and functional recovery of patients is limited due the failure of the body to regenerate the central nervous system (CNS). Since damage resulting from SCI is exacerbated by the immune system's inflammatory response to tissue injury, important efforts are being made to understand how this process occurs and identify new therapeutic targets [2]. In an effort to uncover possible molecular targets for therapy, this study examines the mechanisms by which a specific type of innate immune cell, the macrophage, differentiates into either a pro-inflammatory phenotype that contributes to damage (M1 phenotype) or an anti-inflammatory phenotype with pro-regenerative activity (M2 phenotype).

1.2 - Epidemiology

SCI is estimated to affect approximately 270,000 people in the United States, with an estimated 12,000 new cases annually [1]. Since 2005, approximately 39.2% of SCIs have occurred as a result of automotive accidents, while accidental falls and violent assaults have accounted for the remaining 28.3% and 14.6%, respectively [1]. The average age of SCI patients is 42.6 years with a predominance of SCI occurring in males at 81.8%. Disparities in the incidence of SCI based on race/ethnicity have also been noted, with 67% of SCIs occurring in

Caucasians, 24.4% in African Americans, 7.9% in Hispanics, 0.8% in Native Americans and 2.1% in Asians [1].

SCI presents a significant personal and societal financial burden. A high cervical injury resulting in quadriplegia has a first year cost of $1,044,197 and a lifetime cost of $4,633,137 [1]. The lifetime cost of treatment for a patient suffering from a lower SCI resulting in paraplegia is $2,265,584 after an initial first year cost of $508,904 [1]. Furthermore, the life expectancy of patients suffering from SCI is significantly diminished. Historically, the leading cause of mortality among patients surviving SCI was renal failure; however, advances in urology have significantly reduced renal complications making pneumonia and septicemia the current leading causes of mortality among SCI patients [1]. While there has been an overall improvement in the mortality rate following SCI, there has been little improvement in overall functional recovery.

1.3 - Current SCI Therapies

Treatment options for acute SCI have advanced minimally over the last 20 years. The primary treatment option is high dose methylprednisolone sodium succinate bolus (30mg/kg) administered intravenously within the first 8 hours post-injury followed by continuous infusion for 24 hours (5.4/mg/kg/hour) [3]. Methylprednisolone, a synthetic glucocorticoid, has been shown in some studies to improve the neurologic function and recovery of patients after SCI versus patients receiving placebo [3]. This improved recovery includes improved motor function and improved sensation. Other studies have shown similar results, though the improvements observed were not always considered statistically significant and one study showed no improvement in motor recovery [2-3]. Studies that extended the continuous methylprednisolone infusion from 24 hours to 48 hours initially demonstrated that patients achieved a greater level of

functional motor recovery; however these results were not considered statistically significant after follow-up at 6 months and 1 year. Furthermore, the increased duration (48 hour) of methylprednisolone contributed to an increased rate of infection and sepsis, though no overall increase in the rate of mortality [2-3]. Thus, the immunosuppressive nature of methylprednisolone and other glucocorticoids increases the risk of infection and sepsis with increasing duration of use, which has limited their use as means of recovery for an extended period of time in SCI.

While methylprednisolone acts as an immunosuppressive and anti-inflammatory agent post-SCI, its exact mechanism is not yet fully understood. Methylprednisolone can cross the plasma membrane of affected cells relatively easily due to its characteristic hydrophobicity. Once inside the cell, it is believed that methylprednisolone binds a glucocorticoid receptor, which then migrates into the nucleus and prevents the generation of pro-inflammatory transcription factors such as nuclear factor-kappa B (NF-κB), activation protein-1 (AP-1) and interferon response factor (IRF) family transcription factors [3]. The rationale for treatment is that inhibiting the factors that stimulate cytokine and chemokine production at the site of SCI may be able to reduce secondary tissue damage and peripheral immune cell chemotaxis.

While methylprednisolone is the predominant post-SCI therapy, there have been several recently developed alternatives that are currently under investigation. Riluzole, an FDA-approved treatment for amytrophic lateral sclerosis, is currently undergoing several pre-clinical studies identifying its potential effect on SCI recovery [2]. Riluzole acts as a voltage-sensitive receptor antagonist and also inhibits pre-synaptic calcium-dependent glutamate release. In rat models, studies have reported improved locomotive scores such as the Basso Beattie Bresnahan locomotive score, greater myelin sparing, and overall smaller lesions [2,4]. Cethrin, a Rho-

antagonist that has recently entered clinical trials in humans, has been reported to improve functional recovery and reduce spinal cord lesion after direct application to the dura in mouse and rat models [2,5]. RhoA protein is a calcium-dependent GTPase central to the Rho/ROCK pathway, which inhibits the formation of growth cones on neurons - RhoA inhibition is expected to encourage nerve regeneration [2]. Preliminary analysis of phase I/II open-label trials in humans showed high levels of inter-patient variability. Therefore, establishing efficacy will require a larger study population [2,5]. Finally, another treatment option currently being investigated for SCI is based on surgical decompression of the spinal cord immediately after injury. However this strategy remains controversial. Surgical decompression involves the surgical removal of sections of vertebrae at the site of injury as a method of relieving pressure [2]. Pre-clinical studies in animal models have yielded promising results of recovery post-SCI when decompression was performed within 24 hours post-injury [2,6]. Studies in animal models also noted that earlier decompression generally yielded improved recovery. However, clinical studies of surgical decompression in humans have produced mixed results, further dividing the scientific community on its efficacy [2,6]. Additional studies will need to be completed before its effectiveness can be ascertained. Overall, the limited number and effectiveness of SCI treatments combined with the severe and costly long-term consequences of this condition justify the need to identify new therapeutic targets for intervention. One of the current areas of investigation focuses on understanding the positive and negative effects of immune responses to SCI with the goal of identifying novel therapeutic targets.

1.4 - Immune Response to SCI

SCI occurs over two major phases. If the person survives the primary injury caused by direct trauma, then they must deal with the secondary injury caused by the ensuing immune response to tissue damage [7]. The primary SCI consists of the physical trauma to the spinal cord and the resulting direct tissue damage. This includes the fracturing of vertebrae, severing of neurons and shearing of blood vessels [8]. This phase is accompanied by hemorrhaging, myelin destruction, and cell death. As a consequence of the initial injury, various cellular components that act as danger signals are released from damaged/dying cells [8]. These danger signals, called danger-associated molecular patterns (DAMPs), can include a wide variety of endogenous ligands. DAMPs at the site of SCI include high mobility group box-1 (HMGB1), extracellular mRNA and ATP, hyaluronic acid, heat shock protein (HSP), fibronectin and oxidized lipids [8-11]. CNS resident cells respond to these danger signals and initiate innate inflammatory responses which result in secondary injury.

Microglia, the resident immune cells of the CNS, are capable of responding to DAMPs released at the site of SCI through pattern recognition receptors (PRRs). PRRs recognize a variety of structural motifs that are conserved among pathogens, but similarly respond to DAMPs as a signal of cellular distress. Microglia are known to express a variety of PRRs, including toll-like receptors (TLRs), NOD-like receptors, RIG-like receptors, scavenger receptors and C-type lectin receptors [8]. Other cells resident to the CNS, including neurons, astrocytes, and oligodendrocytes, are also known to express TLRs [8-11]. Activation of TLRs, particularly TLR-2 and TLR-4, can lead to either the MyD88 or TIR-domain-containing adapter-inducing interferon-β (TRIF) signaling pathways which culminate in the activation of pro-inflammatory transcription factors that mediate innate host defense mechanisms, including NF-

κB, AP-1, and IRF [12-13]. NF-κB and AP-1 are responsible for the activation of genes associated with acute inflammation and stimulation of adaptive immunity, including the production of inflammatory cytokines IL-6, IL-8, IL-12, TNF-α, and IL-1β [14]. While this TLR stimulation has been reported to contribute to secondary tissue damage in SCI, deficiencies in either TLR2 or TLR4 signaling also result in exacerbated pathology and diminished functional recovery [15]. IRFs are predominantly responsible for the activation of the antiviral Type I interferons (IFN-α and IFN-β), though their role is not well-defined in the context of SCI. Microglia and oligodendrocytes produce monocyte chemotactic protein-1 (MCP-1), macrophage inflammatory protein-1α (MIP-1α), and stromal-derived factor (SDF) which contribute to the chemotaxis of macrophages from the periphery [16]. Furthermore, these macrophages then obtain entry to the CNS as a result of matrix metalloproteinases (MMPs) and vascular endothelial growth factor (VEGF) secreted by microglia, which aid in the permeabilization of the blood-brain barrier (BBB) [17].

The first responders from the periphery are phagocytes involved in clearing myelin and cell debris. After permeabilization of the BBB, peripheral monocytes infiltrate the CNS approximately 3 days post-injury [18]. Monocytes respond to the cytokines/chemokines secreted by microglia and cells of the CNS and localize to the site of SCI and differentiate into macrophages [20]. Neutrophils also infiltrate the site of SCI and phagocytose myelin, but any additional functions in the context of SCI are less well understood [18]. Macrophages phagocytose myelin and cell debris and release a variety of factors (described in section 1.5) which both enhance inflammation and degeneration. All of these phagocytes contribute to Wallerian degeneration - the extended axonal degeneration that occurs in the axon distal to the transection site. Since neuronal regeneration in the CNS is relatively slow and often incomplete

when compared to the peripheral nervous system, neuronal degeneration in the CNS generally results in long-lasting if not permanent damage and is a main cause of impaired functional recovery in SCI [19-20].

1.5– Macrophages in SCI

Regardless of species or type of injury, intraspinal macrophage accumulation is a prominent and persistent component of experimental and clinical SCI [21-23]. The role of macrophages in SCI has been a topic of debate for many years. Evidence elucidating their role in SCI was often contradictory among studies. Initial research indicated that the presence of macrophages in early SCI was deleterious and exacerbated the injury [20]. However, studies which further investigated the effects of macrophage ablation post-SCI noted a decreased recovery in function pointing to a beneficial role of macrophages [24]. The apparently contradictory effects of macrophages in SCI pointed to the possibility that there were two populations of macrophages, each with its own unique phenotype, acting at the site of injury [25-26]. This observation paralleled *in vitro* studies showing that two different activated macrophage phenotypes can be generated by exposing macrophages to different stimuli [27]. Classically activated macrophages (M1) are generated in response to PRR stimulation in the presence of IFN-γ, while alternatively activated macrophages (M2) are generated in response to the anti-inflammatory cytokine, IL-4 [28]. SCI studies have since shown that M1 macrophages, which cause neurotoxicity and hamper neuroregeneration, persist in SCI lesions for several weeks post-injury [27]. In contrast, M2 macrophages, which support axon growth and are non-neurotoxic, occupy the lesion site for only a few days post-injury [27]. This progressive “loss” of M2 macrophages is thought to be due to the conversion of newly activated microglia and infiltrating

monocytes into M1 macrophages as they respond to pro-inflammatory signaling in the acute SCI environment. Therefore, therapeutic strategies that suppress M1 and enhance M2 macrophages after SCI are actively sought.

Macrophages activated classically are distinguished from alternatively activated macrophages by specific surface markers characteristic of each phenotype. The hallmark indicator of classically activated macrophages is the increased expression of *Nos2*, the gene encoding the enzyme inducible nitric oxide synthase (iNOS). iNOS catalyzes the oxidation of L-arginine into L-citrulline, resulting in the formation of nitric oxide (NO) [28-30]. The recognition of pathogen associated molecular patterns (PAMPs) by macrophages initiates a protective pro-inflammatory response, causing the release of NO intended to damage invading pathogens. However, the release of NO by macrophages at the site of SCI can exacerbate the damage initially caused by the spinal cord trauma. Another marker of M1 macrophages is the upregulation of major histocompatability complex II (MHCII). After a macrophage phagocytoses a pathogen, the macrophage then processes the pathogen in phagolysozomes for presentation to CD4+ T-cells via MHCII [28]. In addition, classically activated macrophages up-regulate the expression of CD86, a costimulatory molecule required for the activation of CD4+ T-cells during antigen presentation. The combined increase in expression of MHCII and CD86 in macrophages bridges the innate immune response with the adaptive immune response. Classically activated macrophages have also been reported to up-regulate the immunoglobulin receptors FcγRIII (CD16) and FcγRII (CD32) [28-29]. Neurons exposed to media from M1 macrophages *in vitro* demonstrate shorter neurite growth and decreased survival [27].

In contrast, when macrophages respond to anti-inflammatory cytokines such as IL-4 and IL-13 they differentiate into M2 macrophages. M2 macrophages are characterized by the

expression of *Arg-1* [16-17]. The specific function of *Arg-1* has not been conclusively established, but its expression appears to be inversely correlated with the expression of *Nos2*. M2 macrophages also upregulate the expression of the mannose receptor (CD206) and YM1, a heparin-binding lectin [16-17,27,30]. The scavenger receptors CD204 and CD163 have also been associated with the M2 macrophage phenotype, targeting low-density lipoproteins (LDLs) and the hemoglobin-haptoglobin complex, respectively [16-17,27,30]. In agreement with the above described release of damaging soluble factors, neurons exposed to media from M2 macrophages *in vitro* exhibit longer neurite growth and sustained survivability [27].

Based on the contradicting results described in the first paragraph of this section, it has been hypothesized that macrophages which arrive at the site of SCI can differentiate into macrophages along a spectrum of phenotypes between two extremes. One of these extremes is the pro-inflammatory, "M1" macrophage that would arise from exposure to the DAMPs released at the site of injury [28]. Classically activated macrophages respond with the release pro-inflammatory cytokines (TNF-α, IL-1β, IL-6) and reactive oxygen species such as the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($HO\cdot$) [28,30]. In addition, M1 macrophages produce substantial quantities of cyclooxygenase-2 (COX2), which is enzymatically responsible for the conversion of arachidonic acid into lipid mediators of inflammation called prostanoids (including prostaglandins and thromboxanes) [17]. These molecules further contribute to the chemotaxis of inflammatory cells, increase vascular permeability, and contribute to pyrogenesis [17]. Further, M1 macrophages can also be detrimental to SCI recovery through the release of neuronal growth inhibitors, including Nogo, myelin associated glycoprotein (MAG), and condroitin sulfate proteoglycan (CSPG). The localized interaction of these inhibitory molecules with microglia and astrocytes contribute to the

formation of a glial scar around the site of SCI [29]. While the purpose of the glial scar is to isolate the primary SCI from the uninjured CNS and prevent further damage, the glial scar also constitutes a physical barrier which prevents robust axonal growth and recovery [29]. Since the DAMPs that induce M1 macrophages continue to be present at the site of injury, classically activated macrophages remain at the site of SCI for an extended period of time contributing to secondary SCI [27].

When macrophages are alternatively activated into anti-inflammatory "M2" macrophages in response to anti-inflammatory cytokines, including IL-4 and IL-13, they release anti-inflammatory cytokines such as IL-10, TGF-β, and IL-1Rα as an immunoregulatory mechanism to moderate inflammatory responses. The cytokine IL-10 acts as inhibitor of M1 macrophages by inhibiting the production of IL-12 and down-regulating the expression of MHCII and costimulatory molecules [32]. Additionally, M2 macrophages secrete TGF-β1, one of three closely related cytokines (TGF-β1, TGF-β2, TGF-β3) [17]. The production of TGF-β1 by alternatively activated macrophages further suppresses the activation of M1 macrophages, thus limiting the ability of M1 macrophages to promote inflammation. In contrast to the pro-inflammatory lipid mediators produced by M1 macrophages, M2 macrophages produce anti-inflammatory lipid mediators including lipoxins, resolvins, and protectins [17,26]. Alternatively activated macrophages also release regenerative mediators which contribute to their role in matrix remodeling and tissue repair. VEGF and platelet derived growth factor (PDGF) are both secreted by M2 macrophages to promote angiogenesis [17]. It is important to note that these alternatively activated macrophages exist as a much smaller population in comparison to classically activated macrophages at the site of SCI, and only transiently remain at the site of injury after the first two weeks post-injury [27]. One of the long-term goals of these studies is to

try to shift the balance of macrophages at the site of SCI from overwhelmingly M1 to M2 in an attempt to reduce inflammation and increase regeneration.

1.6 –miRNA

miRNA are small, non-coding RNA approximately 19-24 nucleotides in length, that regulate gene expression at the post-transcriptional level [36]. miRNA were first described in *Caenorhabditis elegans* by Victor Ambros at Dartmouth College in the early 1990s [37]. miRNA bind the 3' UTR of their complementary target mRNA and either induce the degradation of the mRNA or prevent the mRNA from being translated [36]. The role miRNA play in both healthy host function and disease is a booming field of study. Currently it is estimated that there are more than 2000 miRNA present in humans alone, and that more than one-third of all mRNA are regulated by miRNA [39-40].

miRNA can be encoded within the introns of protein-coding genes and therefore co-expressed with their "host" gene or be independently encoded and transcriptionally regulated [40]. The biogenesis of miRNA involves several steps that start in the nucleus (Fig. 1). RNA polymerase II first transcribes primary-miRNA (pri-miRNA) before undergoing two processing steps involved in miRNA maturation. The first step of miRNA maturation involves the RNAse III enzyme Drosha in conjunction with its partner protein DGCR8. In the nucleus, the Drosha/DGCR8 complex processes pri-miRNA into a 70-nucleotide precursor-miRNA (pre-miRNA) containing a hairpin [36,38-41]. Nuclear channels specific for pre-miRNA (Exportin 5/RanGTP) transport the molecules into the cytoplasm where they undergo the second step in miRNA maturation. The RNAse, Dicer, cleaves the hairpin in pre-miRNA, forming a miRNA duplex [36,38-41]. One strand from the miRNA duplex is loaded into the RNA-induced

silencing complex (RISC) which binds the 3' untranslated region (UTR) of target mRNA. The subsequent translation of the target mRNA is either inhibited by the bound miRNA or the target mRNA is degraded by RNAses in the cytoplasm [36,38-41].

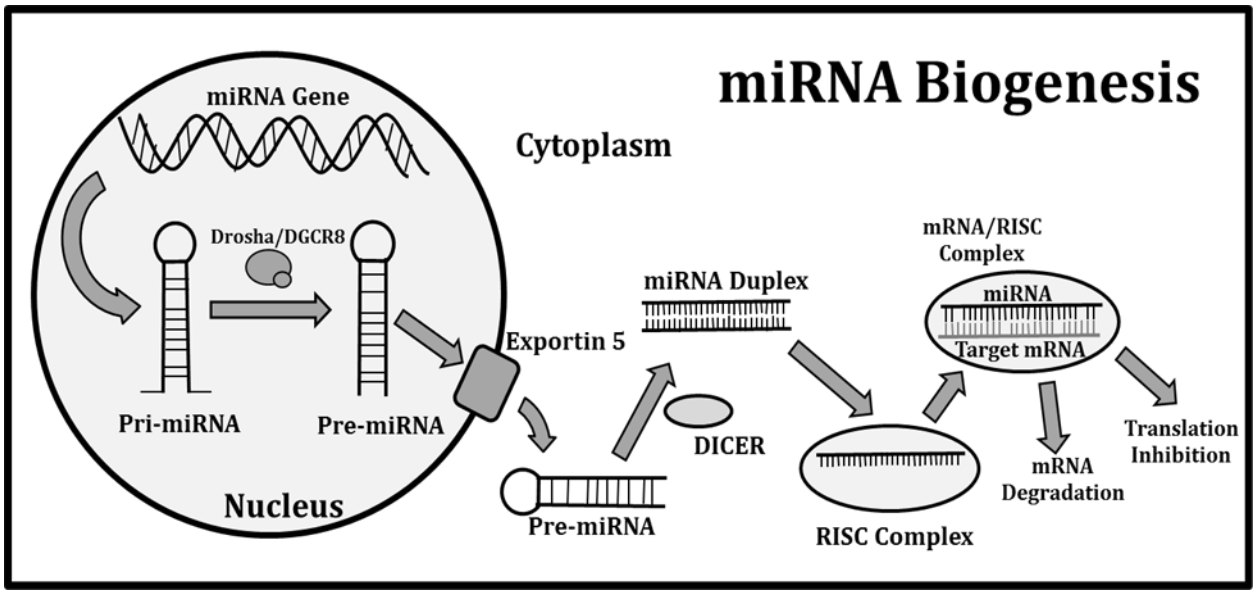

Figure 1. miRNA biogenesis pathway. miRNA are encoded in the genome and transcribed by RNA polymerase II into primary miRNA (Pri-miRNA) which have a hairpin structure with overhangs. Pri-miRNA is then transcribed and processed by the Drosha/DGCR8 complex, which removes the hairpin overhangs, into precursor miRNA (Pre-miRNA). Pre-miRNA exit the nucleus via Exportin 5 and reach the cytoplasm, where they are processed into a miRNA duplex without overhangs by Dicer. The leading strand of the miRNA duplex is then loaded onto the RNA-induced Silencing Complex (RISC) which then joins the miRNA with its target mRNA either inducing degradation or preventing translation.

The role miRNA play in immune regulation and inflammatory conditions has been the subject of numerous studies. Several key regulatory proteins of immune development and function have been shown to be direct targets of miRNA. For example, TNF receptor-associated factor-6 (TRAF6) and IL-1 receptor-associated kinase-1 (IRAK-1), which are key components of the TLR4 signaling pathway, are confirmed targets of miR-146a [42]. It has been demonstrated

that miR-155 plays a vital role in establishing an adaptive immune response to *Salmonella typhimurium* [43]. Mice with deficient bic/miR-155 exhibit impaired B-cell and T-cell function, in addition to defective antigen presentation [44]. miR-155 is also reported to target the transcription factor PU.1 in B cells, which is important in both germinal center response and IgG class switching [40]. Dysregulation in miRNA expression has been linked to an assortment of autoimmune and inflammatory conditions, including systemic lupus erythematosus, multiple sclerosis, and rheumatoid arthritis [45-48]. This thesis explores the possibility that in addition to the roles mentioned above, miR-155 may be important for the shift between M1 and M2 macrophage phenotypes in the spinal cord after injury.

II Materials and Methods

2.1 – Mice

Wild-type (WT) or miR-155 KO (B6.Cg-*Mir155tm1.1Rsky*/J) mice on the C57Bl6/J background originally obtained from Jackson laboratories and kept in specific pathogen-free conditions were used in these studies. All animal experiment procedures were approved under Ohio State University's IACUC protocol # 2009A0036-R1.

2.2 –Cell Culture

To generate bone marrow-derived macrophages (BMDM), the bone marrow cells from femurs and tibias from mice were harvested and cultured as previously described [13]. Briefly, isolated cells were incubated in Dulbecco's Modified Eagle Media (DMEM) supplemented with 10% bovine serum albumin, 1% penicillin/streptomycin, 1% glutamine, and 20% L929 supernatant (containing GM-CSF) in 6-well plates. On day 8 in culture the cells were counted

and replated at 0.5-1.0x10^6 cells per well. Cells received either LPS (10ng/mL) + IFN-γ (20ng/mL) to stimulate the M1 condition, IL-4 (20ng/mL) to stimulate the M2 condition, or media alone to leave the cells undifferentiated (M0). Cells were then harvested at the indicated time-points, generally 24 hours post-stimulation, using Hank's Balanced Salt Solution (HBSS) on day 9 in culture and subsequently washed with PBS before cell lysis for RNA isolation.

2.3 – RNA Isolation

To examine miRNA expression, cells were isolated using the miRvana isolation kit (Ambion) according to manufacturer specifications. Samples were stored at -80 °C until analysis.

2.4 – Reverse Transcription & Real-Time Polymerase Chain Reaction

To determine the relative expression of miRNA, Taqman Real Time PCR (RT-PCR) was used after an initial amplification using miR-155 and sno-202 primers to generate cDNA. Reverse transcription of 10 ng RNA was done using 100mM dNTPs, reverse transcriptase, 10X reverse transcription buffer, and RNAse inhibitor. Reaction mixture was run in a thermal cycler at 16°C for 30minutes, at 42°C for 30 minutes, and at 85°C for 5 minutes. PCR was performed using Taqman universal PCR mix and gene-specific miRNA primers. Reaction mixture was run in RT-PCR machine denaturation step at 95°C for 10 minutes, followed by 40 cycles of denaturation at 95°C for 15 seconds and primer annealing/extension at 60°C for 60 seconds. miR-155 expression was set relative to the housekeeping gene sno202.

The relative gene expression of macrophage markers was determined using SYBR Green or Taqman quantitative RT-PCR. cDNA was generated from 500 ng RNA per sample combined

with random hexamer primers (pN6) and 10mM dNTPs and then incubated at 65°C for 15 minutes. Resulting product was combined with first strand buffer, 0.1M DTT, and RNAse inhibitor and subsequently incubated at 25°C for 2 minutes. Product was combined with Superscript II and the reaction mixture was run in thermalcycler at 25°C for 10 minutes, at 42°C for 50 minutes, and at 70 °C for 15 minutes. Product was combined with 5uM forward and reverse primer of gene of interest and SybrGreen mix or with Taqman mix and Taqman probe before RT-PCR. Expression of target genes was normalized to hypoxanthine guanidine phosphribosyltransferase (HPRT) as a loading control. RT-PCR data was analyzed using the comparative Ct (ΔΔCT) method [49] or the standard curve method [50] depending on whether the test gene and HPRT gene amplification efficiencies were comparable or not, respectively.

2.5. Statistical analysis

Statistical significance was determined using analysis of variance (ANOVA) or unpaired t-test (two-tail, equal SD). Statistical significance was determined to be $p<0.05$. Analysis was completed using GraphPad Prism.

III Results

3.1. Expression of miR-155 is selectively increased in M1 macrophages.

miRNA can regulate the expression of large numbers of genes at the post-transcriptional level. Therefore, specific miRNA expression signatures have been associated with various cellular lineages or phenotypes [50-54]. The expression of a selected group of miRNA reportedly associated with autoimmunity and/or inflammation was quantified in bone marrow

derived macrophages (BMDM) undergoing stimulation with lipopolysaccharide (LPS) and IFN-γ to differentiate into the M1 phenotype or with IL-4 to differentiate into the M2 phenotype (Fig. 2).

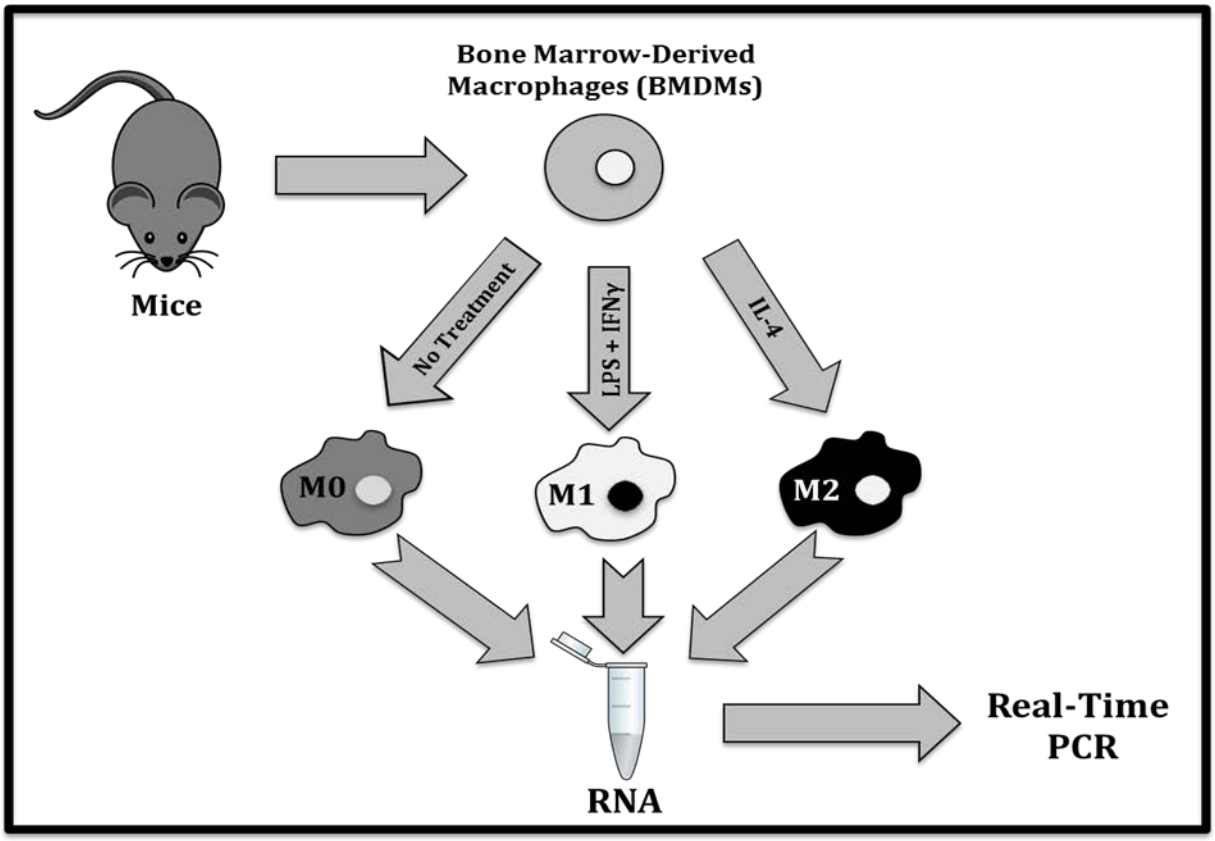

Figure 2. Experimental design. Bone marrow-derived macrophages (BMDMs) were isolated from mice and left in media alone (M0), stimulated with LPS + IFNγ (M1), or stimulated with IL-4 (M2). RNA was isolated and then quantified via Real-Time PCR.

The level of expression 24 hours post-activation was compared to that of M0 macrophages kept in unstimulated but otherwise similar conditions (Fig. 3a). The miRNA chosen for analysis were miR-27b, miR-29b, miR-155, miR-124 and miR-223. miR-27b has been found to be up-regulated in CD4+ T-cells isolated from patients with multiple sclerosis where it has been reported to be a regulator of the differentiation of CD4+ T-cells into Th1 and Th2 phenotypes which are analogous to the M1/M2 phenotypes in macrophages [55]. miR-29b is

another miRNA that has been found to be up-regulated upon T cell activation as a negative feedback loop regulator of Th1 [54]. miR-124 has been reported to be associated with quiescent microglia and miR-223 with blood monocytes [57-58]. Finally, miR-155 was selected due to its association with a variety of inflammatory conditions and responsiveness to TLR stimulation [59-61]. miRNA expression was evaluated by Taqman based Real-Time PCR (RT-PCR) in samples taken 24 hours post-activation. The small RNA sno202 was used as housekeeping normalizing control gene. We observed that, among the miRNAs tested, miR-155 was the most highly up-regulated (fold change (FC)± standard deviation (SD) =182 ±13, post-hoc ANOVA $p< 0.0005$) in M1 conditions while it was not up-regulated in alternatively activated (M2) macrophages (1.0±0.153) (Fig. 3a). These data indicate that miR-155 is associated with M1 but not M2 activation. To better grasp the temporal pattern of miR-155 expression, we performed a time-course analysis of miR-155 expression. The relative expression of miR-155 was quantified at 6, 24, and 48 hours post-stimulation (Fig. 3b). The expression of miR-155 in M1 macrophages had already increased significantly (t test, $p<0.0001$) and reached its maximum observed expression by 6 hours post-stimulation. In contrast, M2 macrophages did not up-regulate miR-155 at any of these time-points. The early and sustained expression of miR-155 led us to hypothesize that that miR-155 plays an important role in initiating the differentiation program of M1 macrophages that may be important in inflammatory responses to SCI.

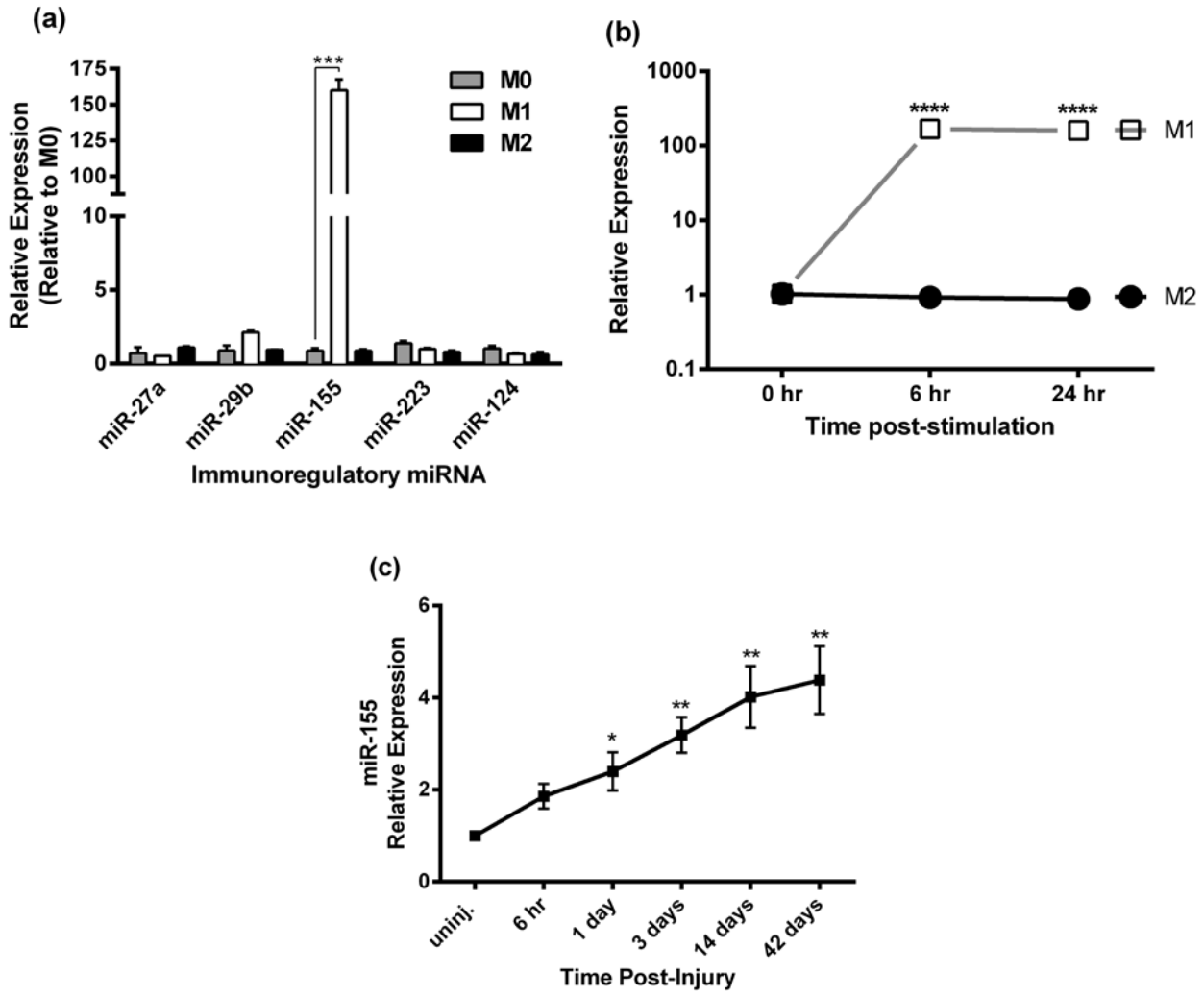

Figure 3. miR-155 is associated with the classically activated macrophage phenotype. Expression of miR-155 was determined by Taqman Real-Time PCR and expressed as mean relative expression (+ SEM) in **(a)** macrophages stimulated *in vitro* for 24 hours in M0, M1, and M2 (n=3) conditions; expression relative to M0 condition;*** Post-hoc ANOVA $p<0.005$ **(b)** M1 and M2 macrophages activated *in vitro* over a 48 hour period; expression relative to 0 hour pre-stimulation time-point; **** unpaired t test $p<0.0005$ or **(c)** SCI tissue collected *in vivo* over a 42-day period; expression relative to uninjured spinal cord tissue; unpaired t-test, *$p<0.05$, **$p<0.005$

3.2. miR-155 is steadily up-regulated in the injured spinal cord tissue.

To ascertain whether miR-155 expression in M1 BMDM is relevant to the inflammatory SCI process *in vivo*, we analyzed miR-155 expression in spinal cord tissue of mice (n=3-5/time-point) that had received a thoracic contusion injury and compared it to that of uninjured spinal cord tissue. The relative expression of miR-155 in tissue isolated from SCI was quantified using RT- PCR (Fig. 3c). The data shows an increase in miR-155 at the site of injury in as early as 6 hours post-injury. The expression of miR-155 in tissue continued to steadily increase until reaching its observed maximum at 42 days post-injury (the last time point measured). Since M1 macrophages accumulate over time in the SCI tissue while M2 macrophages are lost the increase in miR-155 may, at least partially, reflect the accumulation of the miR-155 expressing M1 population [19]. Overall, the observed miR-155 increase in SCI tissue supports the notion that miR-155 plays an important role in SCI inflammation and that miR-155 may be an important therapeutic target in SCI.

3.3. Genetic loss of miR-155 significantly decreases M1 macrophage marker iNOS and TNF-α expression.

In order to determine the extent to which miR-155 contributes to the differentiation program of M1 macrophages, we determined the effect of genetic loss of miR-155 on the M1 phenotype. BMDMs were isolated from miR-155 WT (n=2) or KO (n=2) mice and stimulated into M0, M1 or M2 conditions (n=3 each) for 24 hours. The expressions of two hallmark markers of M1 macrophages, iNOS and TNF-α, were analyzed by RT-PCR (Fig. 4). As expected, iNOS and TNF-α were expressed at a higher level in M1 than M2 WT macrophages. miR-155 deficiency abrogated iNOS expression in M1 macrophages as compared to WT (Fig.

4a, t test $p<0.005$). Similarly, the relative expression of TNF-α was significantly reduced in miR-155 KO M1 macrophages (Fig. 4b, t test $p<0.005$). Neither iNOS nor TNF-α was highly expressed in alternatively activated macrophages, highlighting the non-inflammatory phenotype of M2 macrophages in miR-155-sufficient or deficient macrophages. These data support the hypothesis that miR-155 is necessary for complete manifestation of the characteristic M1 phenotype. However, we wondered whether its loss may additionally impair the M2 macrophage phenotype, which would not be beneficial to neuroregeneration.

3.4. Genetic loss of miR-155 does not impair M2 macrophage marker Arg-1 and IL-13Rα expression.

To determine whether miR-155 loss altered M2 phenotype, we examined the expression of the characteristic M2 marker Arg-1 and of IL-13Rα by RT- PCR in WT (n=2) or miR-155 KO mice macrophages (n=2) exposed to M1 or M2 conditions for 24 hours. Arg-1 was highly expressed in M2 macrophages, as compared to the M1 condition, and was not affected by miR-155 deficiency (Fig. 5a, t test $p>0.05$). WT and miR-155 KO M1 macrophages had very low levels of Arg-1 expression compared with the M2 condition and showed a non-statistically significant increase in miR-155 KO M1 as compared to the WT M1 condition (Fig. 5a, $p = 0.1$). Similarly, miR-155 deficiency did not affect IL-13Rα in either M1 or M2 macrophages (Fig. 5b, $p>0.05$). Overall, these data indicate that a classical M2 marker can still be properly expressed in the absence of miR-155.

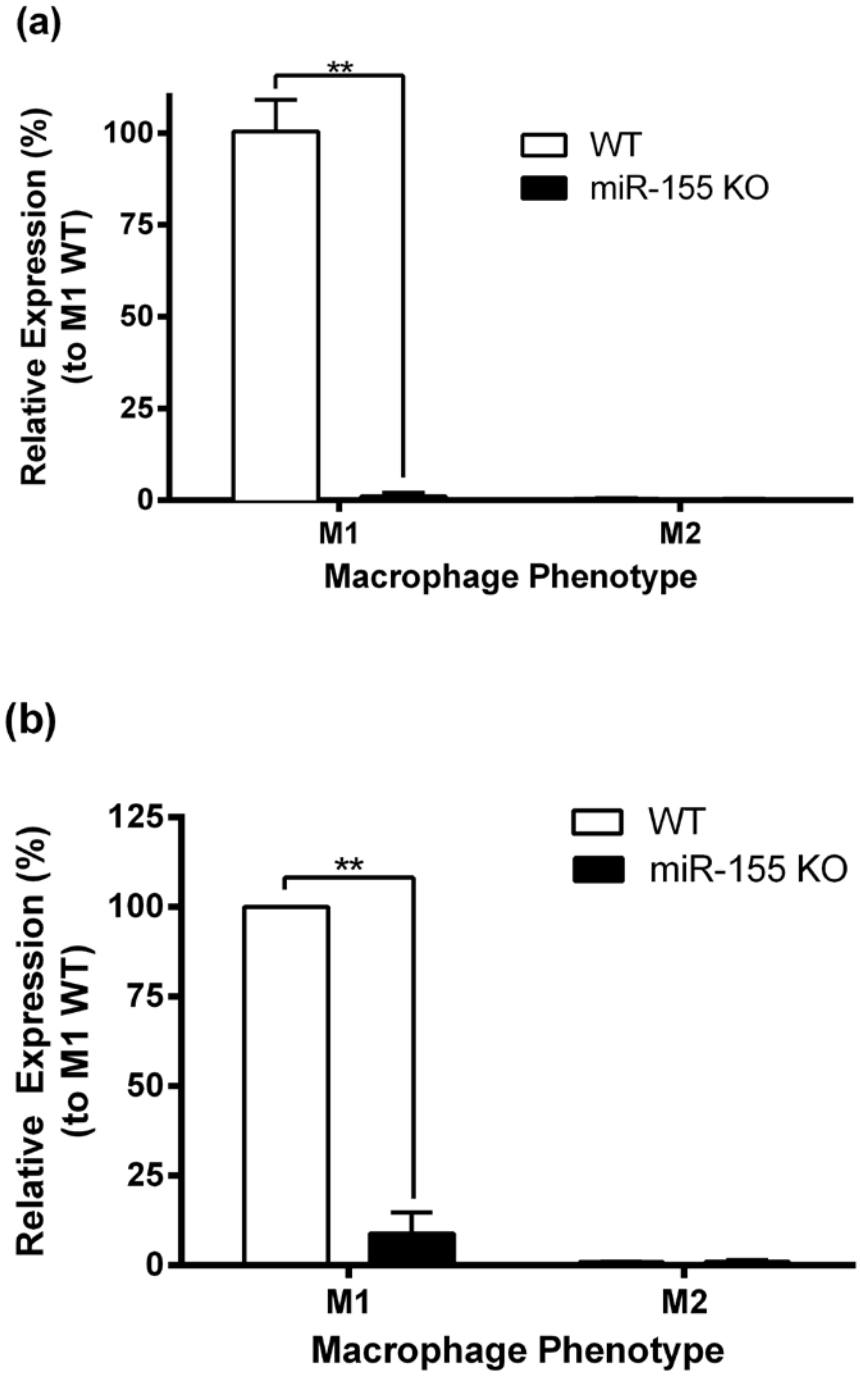

Figure 4. Reduced M1 marker expression in miR-155 knock-out (KO) macrophages. Inducible nitric oxide synthase (iNOS) **(a)** and Tumor Necrosis Factor-α (TNF-α) **(b)** expression was determined by Real-Time PCR in wild-type (WT, n=3) and miR-155 KO (n=3)bone marrow-derived macrophages *in vitro* activated in M1 or M2 conditions for 24 hours. Gene expression is expressed as a percentage +/- SEM of the WT M1 condition. Unpaired t-test, **$p<0.005$

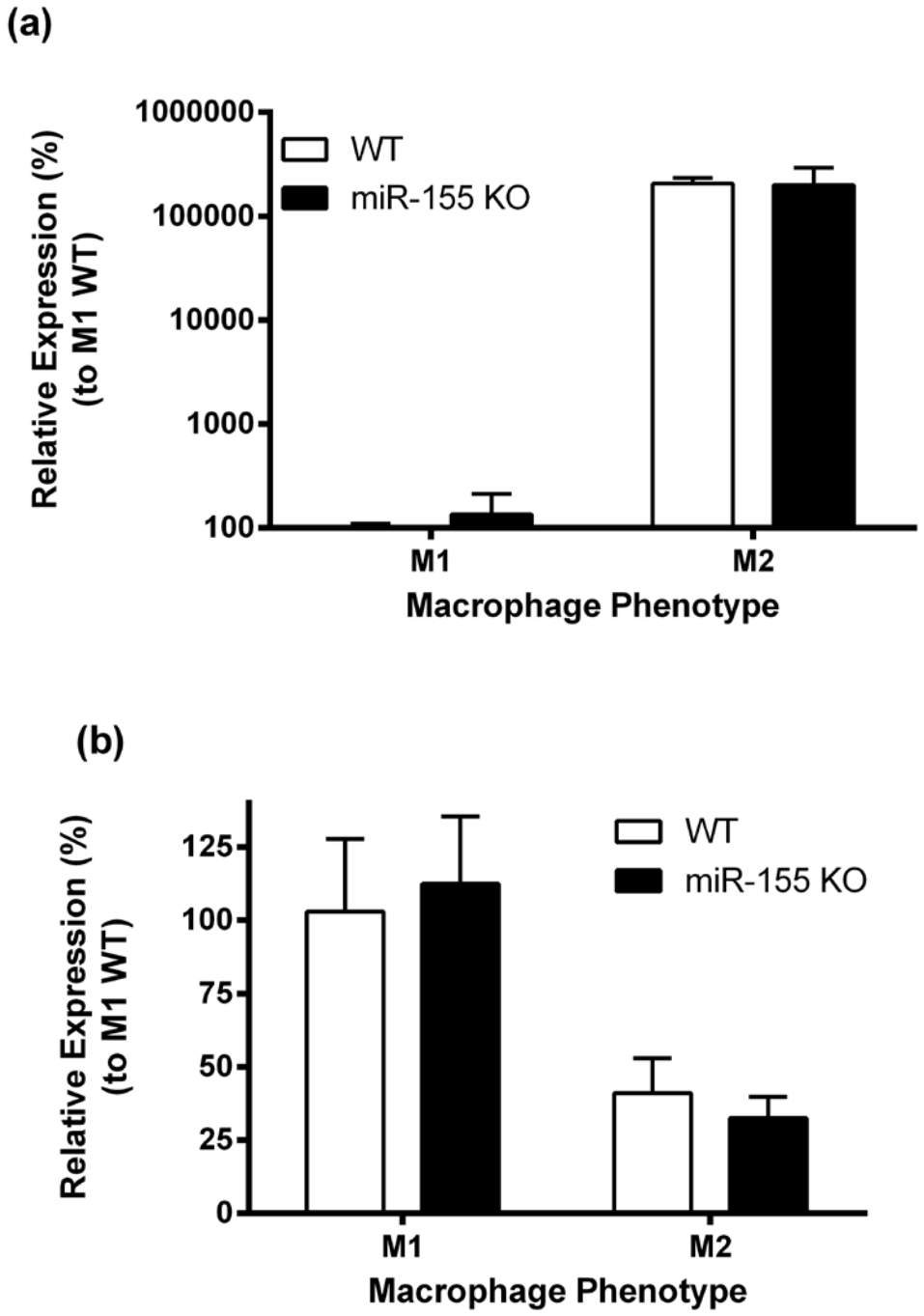

Figure 5. Stable M2 marker expression in miR-155 KO macrophages. Arginase-1 (Arg-1) **(a)** and IL-13Rα **(b)** expression was determined by Real-Time PCR in wild-type (WT, n=3) and miR-155 KO (n=3) bone marrow-derived macrophages *in vitro* activated in M1 or M2 conditions for 24 hours. Gene expression is expressed as a percentage +/- SEM of the WT M1 condition. Unpaired t-test: not significant.

3.5. *In vitro* pharmacologic inhibition of miR-155 reduces M1 macrophage polarization.

The significant decrease in expression of both iNOS and TNF-α in classically activated miR-155 KO macrophages prompted us to examine this pathway as a potential pharmacologic target. Using an oligonucleotide-based inhibitor complementary to miR-155, we examined miR-155 expression in WT BMDMs stimulated in M0, M1, or M2 conditions (n=3) as outlined in Materials and Methods (M&M). As a control, we transfected BMDMs with a scrambled oligonucleotide inhibitor which lacks specificity for miR-155. 24 hours post-activation, we analyzed expression of iNOS and TNF-α by RT PCR. The expression of iNOS in M1 macrophages transfected with miR-155 inhibitor was significantly reduced by 85% as compared to the scrambled inhibitor (Figure 6a, $p<0.05$). Similarly, TNF-α was significantly decreased in M1 macrophages transfected with miR-155 inhibitor as compared to WT (Figure 6b, $p<0.05$). The level of iNOS and TNF-α in undifferentiated macrophages did not exhibit a statistically significant difference in either case. These results suggest that miR-155 is a promising pharmacologic target to limit the pro-inflammatory phenotype in macrophages.

IV Discussion

The poor recovery observed in SCI is the result of a combination of factors, including damage-induced auto-inflammatory mechanisms and inhibited neuronal recovery resulting from growth inhibitors at the site of injury. Furthermore the formation of the glial scar around the site of injury presents a robust barrier which prevents the growth of neurons. These detrimental functions have been linked (though not exclusively) to the pro-inflammatory M1 macrophage which infiltrates the site of injury. Our findings suggest that the extent of polarization in macrophages can, at least in part, be regulated by miR-155.

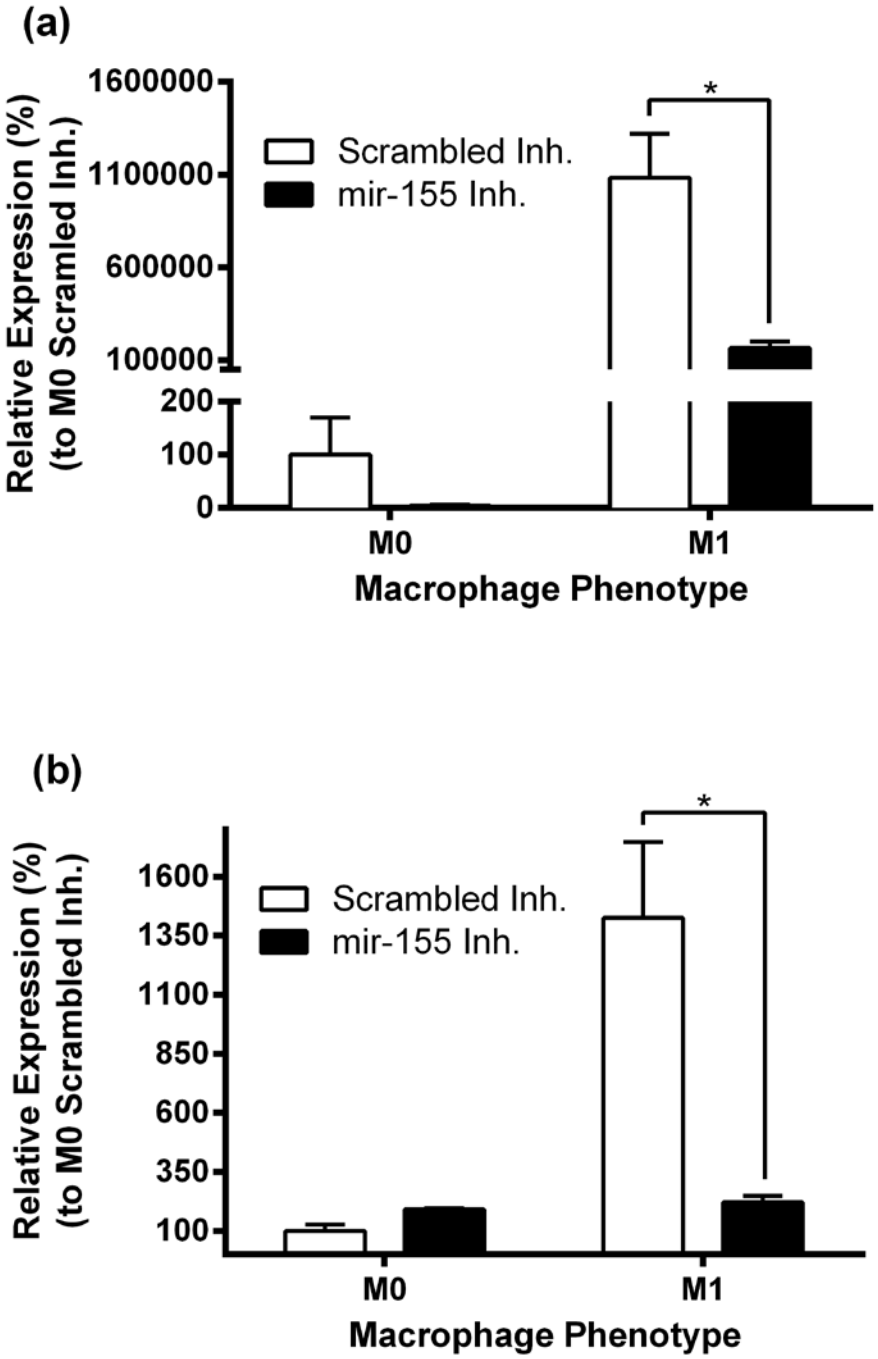

Figure 6. miR-155 inhibitor reduces M1 marker expression. Inducible nitric oxide synthase (iNOS) **(a)** and Tumor Necrosis Factor α (TNF-α) **(b)** expression was determined by Real-Time PCR in wild-type bone marrow-derived macrophages *in vitro* activated in M1 or M2 conditions for 24 hours and transfected with a scrambled (n=3) or a miR-155 oligonucleotide inhibitor (n=3). Gene expression is expressed as a percentage +/- SEM of the scrambled M1 condition. Unpaired t-test, *$p<0.05$

It has been previously shown that neurons exposed to media cultured with M1 macrophages exhibit stunted neurite growth with more branching [19]. Conversely, neurons exposed to media cultured with M2 macrophages show longer neuron growth with less branching [19]. While both show neuron growth, the growth incurred by neurons exposed to M2 media is more appropriate to recovery post-SCI. Furthermore, the neurons exposed to M2 media experience greater survivability compared to neurons exposed to M1 media [19]. The neurons exposed to M1 media yield significantly reduced survivability versus both M2 media and control media. It is therefore critical that the mechanism by which macrophage polarization is regulated is better understood and directed towards an increase in M2 macrophages and a reduction in M1 macrophages.

It has previously been reported that miR-155 may regulate signaling molecules involved in inflammatory pathways [59-60]. Until now, the role miR-155 plays in gene expression in opposed differentiated macrophages had not been previously described. Here, we show that miR-155 is differentially expressed between classically activated and alternatively activated macrophages. The expression of miR-155 increased rapidly and reached a plateau around six hours post-activation in M1 macrophages. The expression of miR-155 in M2 macrophages remained comparatively low and did not demonstrate an increase. In addition, the expression of miR-155 at the site of injury progressively increases up until six weeks post-injury. Furthermore, we show that by knocking out miR-155 in M1 macrophages, we can significantly reduce the expression of iNOS and TNF-α, two key genes involved in inflammation and secondary damage in SCI. Furthermore, the pharmacologic inhibition of miR-155 using oligonucleotide inhibitors significantly reduced both iNOS and TNF-α expression.

The expression of the M2 markers Arg-1 and IL-13Ra seemed to remain stable in miR-155 KO conditions. These results have the important implication that miR-155 is not required for the expression of the M2 phenotype. miR-155 inhibition resulted in observable increased Arg-1 in M1 macrophages at 24 hrs. Although this difference was not significant in the tested conditions, we cannot but wonder whether later time-points may allow an enhanced M2 phenotype to manifest. Since the mere reduction of the M1 phenotype during SCI would be expected to produce an improved environment for regeneration, these results are very promising.

The mechanism by which miR-155 is up-regulated and how it then exerts its effect on inflammatory genes is not entirely understood. However, it seems conceivable that miR-155 up-regulation is a result of TLR stimulation by PAMPs and DAMPs in the microenvironment. Once up-regulated, miR-155 may regulate inflammatory gene expression through various mechanisms. However, one possibility would be through regulation of suppressor of the cytokine signaling (SOCS) family proteins. The SOCS family of proteins contains eight members (SOCS1-7 and CIS) which are important regulators of immune function and cytokine secretion; furthermore, SOCS1 mRNA can be targeted by miR-155 [61]. SOCS proteins are known to negatively regulate Janus kinase/signal transduction and transcription (JAK/STAT) signaling involved in the activation of pro-inflammatory transcription factors. The protein SOCS1 is known to play a role in the regulation of inflammatory pathways and cytokines through the binding of the p65 subunit of NF-κB and inducing its ubiquitin-dependent proteasomal degradation. miR-155 therefore releases the negative regulation of NF-κB by SOCS1, resulting in the transcription of pro-inflammatory cytokines. This model is the basis of current and future studies concerning the role of miR-155 in macrophage polarization and SCI recovery.

The ultimate goal of SCI research is to identify novel therapeutic strategies that can be used in human patients. The observed reduction in M1 markers and inflammatory gene expression associated with miR-155 inhibition or deficiency provides data to support targeting miR-155 as a therapeutic strategy. In devising therapeutic strategies, it is important to consider that macrophages play a critical role in the innate immune system and any alteration in the spectrum of M1/M2 phenotypes should be tightly regulated. For instance, prolonged exposure to cytokines and growth factors released by M2 macrophages has been associated with cancer and neoplasia [62-63]. Additionally prolonged deficiency in M1 macrophages creates vulnerability to bacterial infection, which remains a leading cause of mortality among patients enduring SCI. It is therefore important to be mindful that the modulation of macrophage phenotype should seek to create a more stable balance between M1 macrophages and M2 macrophages rather than to ablate either one. In conclusion, while further investigation is required to determine its mechanism and its effect on neurons and functional recovery, this research illustrates a novel regulator of macrophage polarization and provides a foundational basis for the feasibility of miR-155 inhibition as method of SCI treatment.

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